International Journal of Pharmaceutical Research & Analysis

e-ISSN: 2249 – 7781
Print ISSN: 2249 – 779X

www.ijpra.com

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ETHINYL ESTRADIOL AND LEVONORGESTREL

***Tejal Rathod and Anandkumari D. Captain**

Department of Pharmaceutical Chemistry, A.R.College of Pharmacy and G.H.Patel Institute of pharmacy, Vallabh Vidyanagar, Anand, Gujarat, India - 388120.

ABSTRACT

A simple, accurate, precise, sensitive, specific and reliable stability indicating RP-HPLC method was developed for simultaneous estimation of Ethinyl estradiol (EE) and Levonorgestrel (LEV) in Pharmaceutical dosage form. The developed method with mobile phase Acetonitrile: Water (75: 25), Analytica brownee C-18 (150×4.6 mm, 3µm particle size) as a stationary phase and flow rate was 0.8 ml/min. Detection was carried out at 230 nm in PDA detector. The calibration curve of Ethinyl estradiol and Levonorgestrel was found to be linear in the range of 4-14 µg/ml and 20-70 µg/ml respectively. The proposed method has been validated for precision, accuracy, robustness. As the proposed method can effectively separate the drugs from all their degradation products, it can be employed as stability indicating method.

Keywords: Ethinyl estradiol, Levonorgestrel, High Performance Liquid chromatography, Validation.

INTRODUCTION

Ethinyl estradiol- 19-nor-17α-pregna-1,3,5(10)-trien-20yne-3,17β-diol is semi synthetic steroid and Levonorgestrel - 13β-ethyl-17β-hydroxy-18,19-dinor-17α-Pregn-4-en-20-yn-3-one is oral progestin. Structure of Ethinyl estradiol and Levonorgestrel is shown in Fig.1 and Fig. 2 [1-6]. They are used as oral contraceptive for human. This Combination is official in IP-2010, U.S.P-25; N.F.-30, B.P.-2010 [1-3]. As per literature survey methods like UV-spectrophotometric [14, 15, 18], HPLC [8, 9, 11-13, 16, 17, 19-21], ELISA [10] have been reported for simultaneous estimation of Ethinyl estradiol and Levonorgestrel. But there is no any method have been reported for stability indicating RP-HPLC method for simultaneous estimation of both the drugs in pharmaceutical dosage form. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, etc. and separation of drug from degradation Products. This work presents stability indicating RP-HPLC method for the simultaneous determination of Ethinyl estradiol and Levonorgestrel in bulk and pharmaceutical dosage form.

MATERIALS AND METHODS

Standard Ethinyl estradiol and Levonorgestrel were obtained as gift sample from Famycare Ltd., Ahmedabad and Unicure Remedies Pvt. Ltd., Vadodara respectively. Perkin Elmer-200 (gradient) chromatograph with PDA detector was used with Total Chrom Workstation (Ver.6.3.1) Software. Acetonitrile - HPLC grade, Water - HPLC grade, Lichrosolv, Merck India Ltd.,Mumbai, was used. A commercial tablet formulation Dear-21 was purchased from local market.

Selection of Detection wavelength

Solution of 100 ppm of each EE and LEV were prepared, and scanned over the range 200-400 nm and the spectra were recorded. Wavelength 230 nm (at which both the drugs showed good absorbance) was selected as a

Corresponding Author:- Tejal Rathod **Email:-** rathodtejal36@gmail.com

detection wavelength.

Selection of Mobile phase

After trials of various mobile phase compositions, ACN: H_2O (75:25 v/v) is selected for the estimation. Chromatogram in optimized mobile phase is shown in Fig. 3.

Preparation of standard and stock solution

Stock solution of the drugs prepared by dissolving 25 mg of Ethinyl estradiol and Levonorgestrel with 5 ml Acetonitrile in 25 ml volumetric flask and diluted with mobile phase up to the mark. From this stock solution, pipette out aliquots from stock solution and standard solution of Ethinyl estradiol and Levonorgestrel of 100 µg/ml and 500 µg/ml respectively.

Optimized Chromatographic Conditions

ParameterOptimized condition

Instrument: Perkin Elmer HPLC systemwith Total Chrom Workstation (Ver.6.3.1) Software

Column: Perkin Elmer LC- C18 column (150 X 4.6 mm, I.D. 3 µ)

Mobile phase: ACN: H2O (75:25v/v)

Flow rate: 0.8 ml/min

Detection: 230 nm

Injection volume: 20µl

Temperature: 25 ºC

Calibration of standards

Calibration curve of EE and LEV were prepared for concentration range of 4-14 µg/ml (EE) and 20-70 µg/ml (LEV) were prepared by pipette out different volumes from each stock solution and dilute up to the marks with mobile phase.

METHOD VALIDATION

Linearity

Calibration curve of EE and LEV were chromatographed over the range of 4-14 µg/ml and 20-70 µg/ml respectively. The calibration curve were linear and regression analysis were obtained. Linearity plots were shown in Fig. 5 and Fig. 6. Results for linearity are shown in table 3.

Accuracy (Recovery study)

Accuracy of an analysis is determined by calculating systemic error involved. It was determined by calculating recovery of both the drug by standard addition method at three different concentration levels of drug. Accuracy was determined at three different level 80 %, 100 % and 120 % of the target concentration 10 µg/ml of EE and 50 µg/ml of LEV in triplicate and calculating % recovery. Results are shown in table 4.

Precision

Repeatability was assessed by analyzing six injection of a homogeneous sample of 6 µg/ml of EE and 30 µg/ml of LEV. Intra-day precision was performed using three different concentration 6 µg/ml, 8 µg/ml, 10 µg/ml for EE and 30 µg/ml, 40 µg/ml, 50 µg/ml for LEV in triplicate at three different time interval in a day.

Inter-day precision was performed using three different concentration 6 µg/ml, 8 µg/ml, 10 µg/ml for EE and 30 µg/ml, 40 µg/ml, 50 µg/ml for LEV in triplicate for three consecutive days. (Table 5 & 6).

LOD and LOQ

LOD and LOQ of the drug were derived by calculating the signal-to-noise ratio (i.e. 3.3 for LOD and 10 for LOQ) using the 4, 6 and 8 µg/ml of EE and 20, 30 and 40 µg/ml of LEV. The results were shown in table 7.

Robustness

Robustness of the method was carried out by deliberately made small variation in the flow rate (± 0.2 ml/min.), organic phase ratio (±2%), by using 10 µg/ml of EE and 50 µg/ml of LEV. The results were shown in table 8.

System suitability

It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. For this, parameters like Plate number (N), Resolution (R), tailing factor, Capacity factor, HETP, Peak symmetry of samples were measured. The results were shown in table 9.

Specificity

Commonly used excipients in tablet preparation were spiked in a pre-weighed quantity of drugs and then area was measured and calculations carried out to determine the quantity of the drugs.

Assay of marketed formulation

Twenty tablets were accurately weighed, average weight was determined and ground to fine powder. A quantity of powder equivalent to 5 mg (EE) and 25 mg (LEV) was transferred into 10 mL volumetric flask containing 5 ml of Mobile phase, sonicated for 10 min and diluted to mark with same solvent to obtain 500 µg/ml of EE and 2500 µg/ml of LEV. The resulting solution was filtered using 0.45 µm filter (Millifilter, MA). Solution containing EE 10 µg/ml and LEV 50 µg/ml was prepared from above solution. 20 µl of the test solution was injected and chromatogram was recorded under optimized chromatographic condition and peak area was measured. The assay procedure was made in triplicate and % drug was calculated. Results are shown in table 10. Chromatogram is shown in Fig. 6.

Forced degradation

Acid degradation

Accurately weighed tablet powder equivalent to 5 mg of EE and 25 mg of LEV and transferred to a 250 ml round bottom flask, to this add 5 ml HPLC grade Acetonitrile, dissolve it and add 5 ml 0.1 N HCl. The mixture was refluxed at 40ºC for 2 hours. Then, solution was neutralized with NaOH solution to avoid further degradation. The forced degradation was performed in the dark to exclude the possible degradation effect of light. From above stock solution prepare solution containing 10 µg/ml EE and 50 µg/ml of LEV and further analysed as per methodology. (Fig. 7)

Base degradation

Accurately weighed tablet powder equivalent to 5 mg of EE and 25 mg of LEV and transferred to a 250 ml round bottom flask, to this add 5 ml HPLC grade Acetonitrile, dissolve it and add 5 ml 0.1 N NaOH. The mixture was refluxed at 60ºC for 2 hours. Then, solution was neutralized with HCl solution to avoid further degradation. The forced degradation was performed in the dark to exclude the possible degradation effect of light. From above stock solution prepare solution containing 10 µg/ml EE and 50 µg/ml of LEV and further analysed as per methodology. (Fig. 8)

Oxidative degradation

Accurately weighed tablet powder equivalent to 5 mg of EE and 25 mg of LEV and transferred to a 25 ml volumetric flask, to this add 5 ml HPLC grade Acetonitrile, dissolve it and add 5 ml 1% H_2O_2. The sample solutions were stored at 25ºC (room temp.) for 30 minutes. Then solution is diluted with Mobile phase up to the mark. From above stock solution prepare solution containing 10 µg/ml EE and 50 µg/ml of LEV and further analysed as per methodology. (Fig. 9)

Thermal degradation

Accurately weighed tablet powder equivalent to 5 mg EE and 25 mg LEV (7.9 gm) was taken in porcelain dish and exposed to a temperature of 80ºC for 6 hour in hot air oven. After 6 hour, sample powder was transferred to a 25 ml volumetric flask, dissolved in 5 ml HPLC grade Acetonitrile and diluted up to the mark with Mobile phase. Then solution is diluted with Mobile phase up to the mark. From above stock solution prepare solution containing 10 µg/ml EE and 50 µg/ml of LEV and further analysed as per methodology.

Photolytic degradation

Accurately weighed tablet powder equivalent to 5 mg EE and 25 mg LEV (7.9 gm) was taken in petri-dish and exposed to UV light (UV=200 W h/m^2) (ICH Q1B, Option II) in a photo-stability chamber for 24 hour. After 24 hour, sample powder was transferred to a 25 ml volumetric flask, dissolve in 5 ml HPLC grade Acetonitrile was diluted with Mobile phase up to the mark. From above stock solution prepare solution containing 10 µg/ml EE and 50 µg/ml of LEV and further analysed as per methodology.

RESULT AND DISCUSSION

The present work aimed development and validation of stability indicating RP-HPLC method for simultaneous estimation of EE and LEV. Method was developed in mobile phase ACN: H2O (75:25v/v). Detection was carried out at 230 nm. Method was validated as per ICH guidelines. Linearity and regression data were shown in table 3 and Fig.4, 5. % recovery for EE and LEV were within the range (98% - 102%). Results were shown in table 4. So, the developed method is accurate. %RSD values were <2 for repeatability, intra-day and inter-day precision. Results were shown in table 5 and table 6. So, the developed method was found to be precise. LOD and LOQ values were shown in table 7. So, the developed method was found to be sensitive. Small changes were carried out in mobile phase and flow rate for robustness study, in that % RSD of area was found to be <2. Results were shown in table 8.So, the developed method was found to be robust. Various forced degradation conditions were performed in proposed method and it can efficiently separate all the degradation products from the drugs. % degradation values are 5% to 20% degradation of the drug substance, have been considered as reasonable and acceptable for validation of chromatographic assays. Results were shown in table 11. So, the developed method is stability indicating.

Table 1. Linearity data for EE

CONCENTRATIONS(µg/ml)	AREAMEAN ± S.D. (n=6)	% RSD
4	152167.19 ±1838.04	1.2079
6	236132.86 ± 2581.26	1.0931
8	312503.57 ± 2844.00	0.9100
10	406188.29 ± 3297.23	0.8117
12	494997.48 ± 2459.29	0.4968
14	603180.49 ± 4125.56	0.6839

Table 2. Linearity data for LEV

Concentrations (µg/ml)	Area Mean ± S.D. (n=6)	% RSD
20	1033936.36 ± 14080.25	1.3618
30	1423533.55 ± 17775.60	1.2486
40	1925034.27 ± 17853.91	0.9274
50	2258812.90 ± 19172.01	0.8487
60	2674226.04 ± 20156.11	0.7537
70	3136240.33 ± 13808.24	0.4402

Table 3. Statistical data for EE and LEV

Parameter	EE	LEV
Linearity [µg/ml]	4-14	20-70
Linearity Equation	y = 44671x – 35643	y =42128x + 176854
Slope	44671	42128
Intercept	35643	176854
Correlation Coefficient (R^2)	0.997	0.9985

Table 4. Recovery study of EE and LEV

Conc. of Sample taken [µg /ml]	Level	Conc. of Pure API spiked [µg /ml]	Total Conc. [µg /ml]	Mean Total Conc. Found (n=3) [µg /ml]	% Recovery Mean (n=3)	% RSD
EE 10	80%	8	18	18.09	100.52	0.7772
	100%	10	20	20.03	99.27	0.2253
	120%	12	22	21.95	99.60	1.2141
LEV 50	80%	40	90	88.85	98.72	0.6156
	100%	50	100	100.19	100.19	0.8184
	120%	60	110	109.79	99.79	1.6019

Table 5. Repeatability data of EE and LEV

Concentration	EE (6 µg/ml)	LEV (30 µg/ml)
Area	235373.21	1414577.29
	236459.49	1425345.29
	239652.71	1405963.19
	240193.57	1451629.18
	233426.38	1445349.12
	238124.24	1439871.29
Mean	237204.93	1430455.89
± SD	2606.03	18093.68
% RSD	1.0986	1.2648

Table 6. Inter-day and Intra-day Precision data of EE and LEV

Concentration (µg/ml)	Intra-day Area Mean (n=3) ± SD	% RSD	Inter-day Area Mean (n=3) ± SD	% RSD
EE				
6	235943.15 ± 766.98	0.3250	235167.53 ± 3176.51	1.3507
8	310966.70 ± 614.52	0.1976	311556.05 ± 2042.99	0.6557
10	403768.21 ± 512.62	0.1269	403361.66 ± 1216.47	0.3015
LEV				
30	1416919.69 ± 2452.13	0.1730	1422895.59 ± 17181.58	1.2075
40	1916495.94 ± 2044.71	0.1066	1930501.48 ± 19173.80	0.9932
50	2263916.67 ± 6271.14	0.2770	2255674.70 ± 17280.28	0.7660

Table 7. LOD and LOQ of EE and LEV

DRUG	LOD [µg /ml]	LOQ [µg /ml]
EE	0.25	0.75
LEV	2.46	7.47

Table 8. Robustness data for EE and LEV

Concentration of Sample taken [µg /ml]	Parameter	Area Mean (n=3) ± SD	% RSD
EE 10	organic phase 73:27	404852.23 ± 1143.11	0.2823
	organic phase 77:23	403752.51 ± 1359.18	0.3366
	Flow rate 0.6 ml/min	403557.33 ± 2322.96	0.5756
	Flow rate 1.0 ml/min	404314.5 ±1880.7	0.4651
LEV 50	organic phase 73:27	2256191.53±21213.15	0.9402
	organic phase 77:23	2237617.19±14051.8	0.6279
	Flow rate 0.6 ml/min	2258497.33±15825.74	0.7007
	Flow rate 1.0 ml/min	2247106.99±15228.12	0.6776

Table 9. System suitability data for the developed method

Systemsuitability parameter	Result of proposed method		Acceptance criteria
	EE	LEV	
Retention time (min.)	2.73	3.61	
Theoretical plate number	10934	16906	> 2000
Resolution	3.00		> 2
Tailing factor	1.48	1.4	< 1.5

Table 10. Assay of marketed formulation

Parameter	Tablet formulation	
	EE	LEV
Concentration [µg /ml]	10	50
Concentration found [µg /ml] *	9.93 ± 0.0579	50.09 ± 0.3109
%Purity	99.3 %	100.18 %
%RSD*	0.5834	0.6208
Limit[1-3]	NLT 110%	NLT 90%

(* denotes average of Three determinations)

Table 11. Stability data of EE and LEV

Condition	Optimized degradation condition	% Degradation		No. of Degradation products	
		EE	LEV	EE	LEV
Acidic	0.1 N HCl, 40ºC, refluxed for 2 hr	16.82%	8.95%	1	1
Alkaline	0.1 N NaOH, 60ºC, refluxed for 2 hr	7.21%	13.32%	1	1
Oxidative	1%H2O2, room temp, 30 min	12.16%	-	1	-
Thermal	80ºC, 6 hr	-	-	-	-
Photolytic	UV light 200 W h/m^2 ,24 hr	-	-	-	-

Table 12. Summary of RP-HPLC method

Parameters	EE	LEV	REMARK
Linearity (µg/ml)	4 - 14	20 - 70	Linear
%Recovery (%)	99.27 - 100.52	98.72 - 100.19	Accurate(98.0%-102%)
Precision(%RSD) Repeatability (n=6) Intra-day (n=3)Inter-day (n=3)	1.0986 0.1269- 0.3250 0.3015- 1.3507	1.2648 0.1066- 0.2770 0.7660- 1.2075	Precise (%RSD < 2)
LOD (µg/ml)	0.25	2.46	
LOQ (µg/ml)	0.75	7.47	
Robustness	Robust	Robust	Robust(No difference in result)

Fig 1. Structure of Ethinyl estradiol

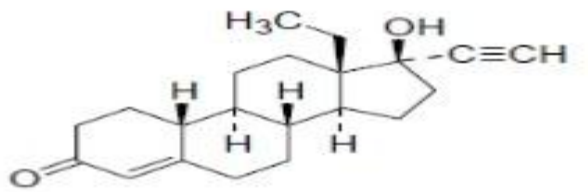


Fig 2. Structure of Levonorgestrel

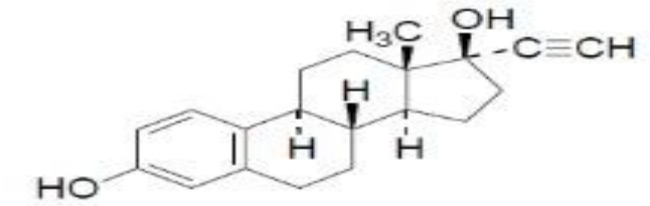


Fig 3. Chromatogram of Ethinyl estradiol and Levonorgestrel in optimized chromatographic condition

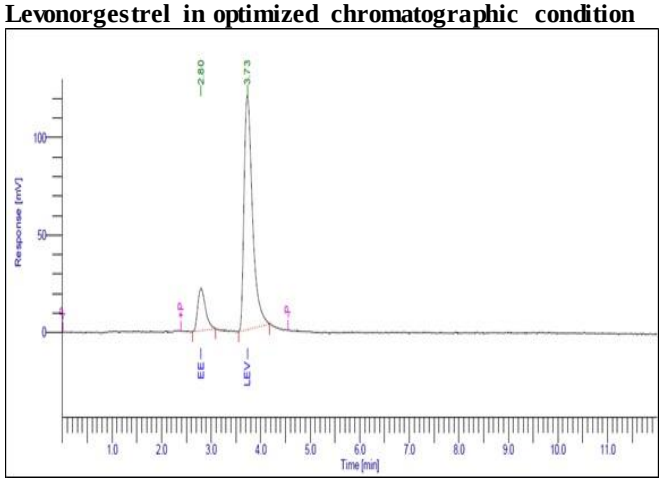


Fig 4. Calibration curve of EE

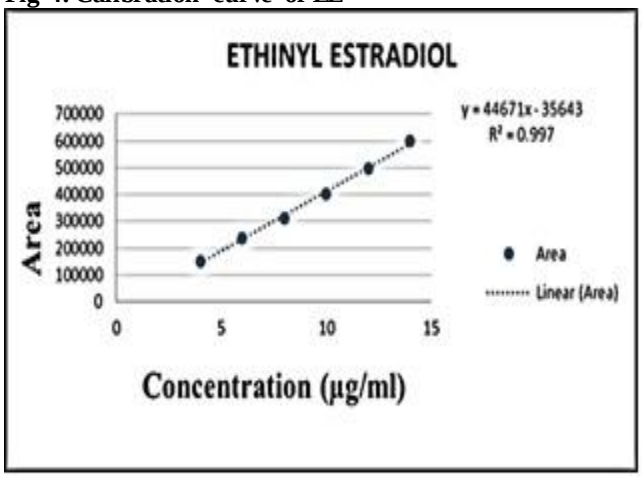


Fig 5. Calibration curve of LEV

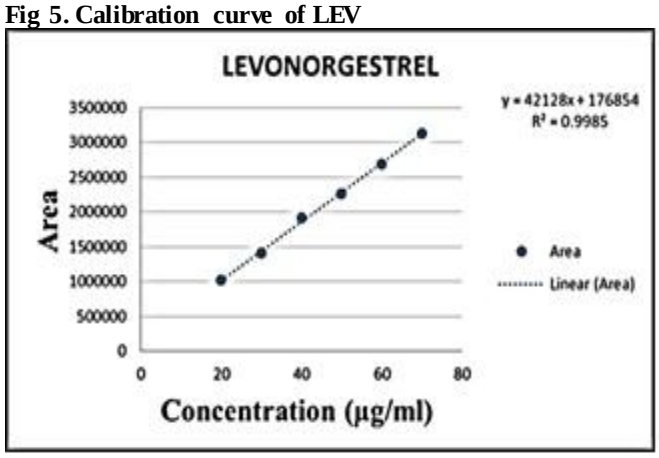


Fig 6. Chromatogram of Dear-21 tablet solution containing 10 µg/ml of each Ethinyl estradiol and 50 µg/ml Levonorgestrel using optimized mobile phase

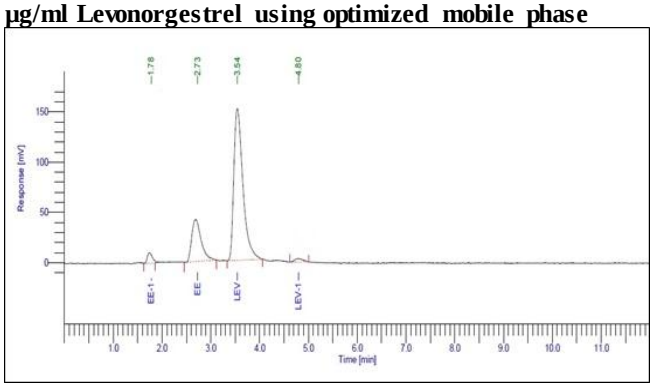


Fig 7. Chromatograph of Ethinyl estradiol (10 µg/ml) and Levonorgestrel (50 µg/ml) (tablet) and its degradation products in the acid degradation study

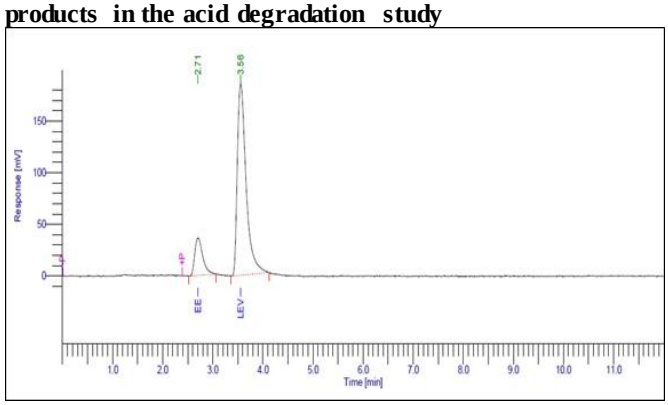


Fig. 8. Chromatograph of Ethinyl estradiol (10 µg/ml) and Levonorgestrel (50 µg/ml) (tablet) and its degradation products in the base degradation study

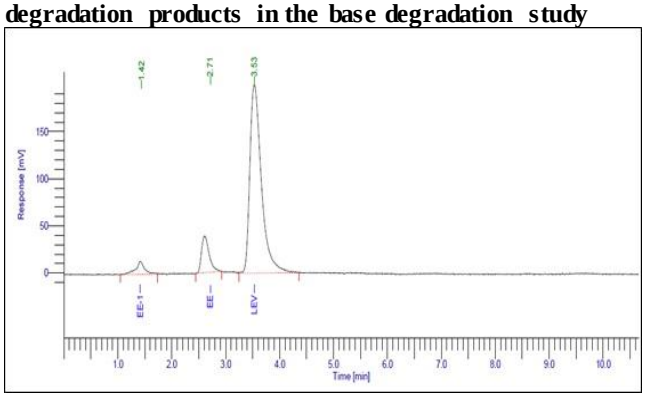
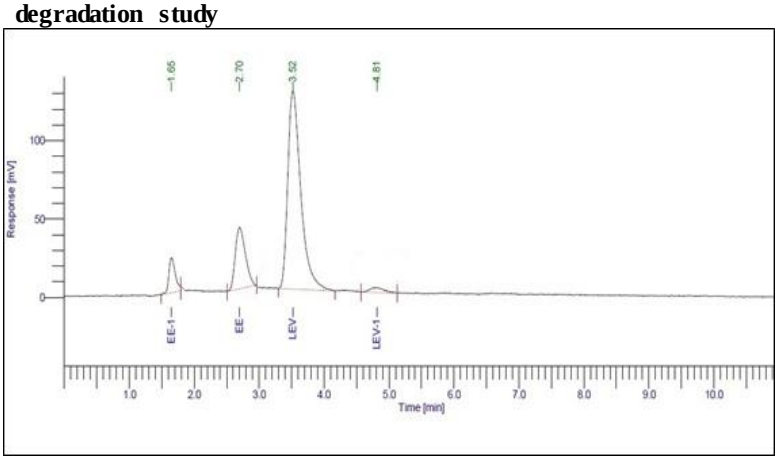


Fig 9. Chromatograph of Ethinyl estradiol (10 µg/ml) and Levonorgestrel (50 µg/ml) (tablet) and its degradation products in the oxidative degradation study



CONCLUSION

Stability indicating RP-HPLC method for simultaneous estimation of EE and LEV was developed and validated as per ICH guidelines. The developed method was found to be accurate and precise with % RSD <2%. So, it can be conclude that the developed method is simple, accurate, precise, sensitive and robust. As the % degradation of drug substance were between 5%-20%, the developed method was found to be stability indicating

ACKNOWLEDGEMENT

We are heartly thankful to Famycare Ltd., Ahmedabad and Unicure Remedies Pvt Ltd., Vadodara for providing EE and LEV as gift sample respectively. We are also thankful to Sophisticated Instrumentation Center for Applied Research and Testing (SICART), Vallabh Vidyanagar.

REFERENCES

1. Indian Pharmacopoeia. The Ministry of Health and Family welfare, Indian Pharmacopoeial Commission, Ghaziabad. II, III, 2010, 1306-1307, 1581-1583, 2783, 2795.
2. United State Pharmacopeia. The United States Pharmacopoeial Conventional. II, III, 2011, 2771, 3298.
3. British Pharmacopoeia. Health Minister on the recommendation of the Commission on Human Medicines, 6th ed. II, III, 2010, 833-834, 1269.
4. International Pharmacopoeia. CBS Publishers and Distributors, New Delhi, India, 3rd ed, 1991, 121-122,166-167.
5. Matindale. The extra pharmacopoeia, published by Royal Pharmaceutical Society of Great Britain, 30th Edition, 1164-1170.
6. The MERCK Index, Merck research Laboratories, U.S.A., 14th ed, 6704, 3734.
7. Satinder A, Stephen S. Handbook of Modern Pharmaceutical Analysis, 3rd edition, Academic Press Harcourt Place, 32 Jamestown Road, London, 2001, 445-483.
8. Strusiak SH *et al*., Determination of Ethinylestradiol in solid dosage forms by High-Performance Liquid Chromatography. *Journal of Pharmaceutical Science*, 71, 1982, 636-640.
9. Patel MG, Vidya Sagar G. Development And Validation Of Analytical Method For Simultaneous Estimation Of Ethinylestradiol And Cyproterone Acetate In Combined Solid Dosage Form. *International journal of Universal Pharmacy and Life Science*, 2, 2012, 612-622.
10. Chun P, Ya-Feiwu, Hong Y. Trace analysis of contraceptive drug Levonorgestrel in wastewater samples by a newly developed indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA) coupled with solid phase extraction *Analytica Chimica Acta*, 628, 2012, 73-79.
11. Tao T, Pingliang L, Laixin L, Dazhao S, Jianqiang L, Yongsong C. Development and validation of a HPLC method for determination of Levonorgestrel and Quinestrol in rat plasma, *Biomedical Chromatography*, 24, 2010, 706-710.
12. Durga Prasad S, Changala Reddy G, Prasad PSS, Mukkanti K. Simultaneous HPLC estimation of Levonorgestrel and Ethinylestradiol. *International Journal of Pharmaceutical Science*, 2004, 231-234.
13. Ravindra A, Hima P, Narayana Swamy K, Vinod Kumar K. Validated RP- HPLC Method for Simultaneous Estimation of Levonorgestrel and Ethinylestradiol Combined Dosage Form. *Journal of Scientific & Innovative Research*, 2, 2013, 598-607.

14. Berzas JJ, Nevada J, Rodriguez Flores G, Castaiieda P. Simultaneous Spectrophotometric Determination of Ethinylestradiol and Levonorgestrel by Partial Least Square and Principle Component Regression Multivariate Calibration. *Analytica Chemica Acta*, 1997, 257-265.
15. Ahmadia F, Ghordoie R.Determination of Levonorgestrel and Ethinyl Estradiol inPharmaceutical Formulations by H-Point Standard Addition Method in Nonaqueous Solvent Using Simultaneous Addition of Both Analytes. *Journal of Reports in Pharmaceutical Sciences*. 1, 2012, 7-18.
16. Arsova-Sarafinovska Z, Liljana U, Katerina S, Dragan D, Aneta D. Determination of Ethinylestradiol and Levonorgestrel in oral contraceptives with HPLC methods with UV detection and UV/fluorescence detection. *Macedonian pharmaceutical bulletin*, 2006, 52, 9-16.
17. Prabhakar B, Deshpande S. Simultaneous Estimation of Ethinylestradiol And Levonorgestrel From Transdermal Patches By HPLC. *Indian Journal of Pharmaceutical Science*, 61, 1999, 12-15.
18. Berzas JJ *et al*., Simultaneous determination of Ethinylestradiol and Levonorgestrel in oral contraceptives by derivative spectrophotometry. *Analyst*, 122, 1997, 41-46.
19. Reif VD, Eickhoff WM, Jackman JK, DeAngelis NJ. Automated stability-indicating high-performance liquid chromatographic assay for Ethinylestradiol and Levonorgestrel tablets. *Pharma res*, 4, 1987.
20. Sarat M, Rambau C. A validated simultaneous RP-HPLC method for determination of Desogestrel and Ethinyl estradiol tablets. *International journal of pharmacy and pharmaceutical science*, 4, 2012, 115-119.
21. Praveen C, Rangnath MK, Divakar P. Method Development and Validation for Simultaneous Estimation of Ethinyl Estradiol and Drospirenone and Forced Degradation Behavior by HPLC in Combined Dosage Form. *Pharmaceutica Analytica Acta*, 4, 2013, 1-5.